# TRANSLATION


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(54) Title of the Invention
Modified tissue plasminogen activator and the preparation method


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## SPECIFICATION

1. Title of the Invention

Modified tissue plasminogen activator and the preparation method

2. Patent Claims

(1) A modified tissue plasminogen activator carrying, on the amino group of an amino acid residue on the tissue plasminogen activator, a polyethylene glycol unit, which is activated using an active group, by connecting the amino group of the amino acid residue on the tissue plasminogen activator to the terminal hydroxyl group of the polyethylene glycol represented by general formula (I),

$$R_1\text{-}(OCH_2CH_2)_n\text{-}OH \qquad \text{(I)}$$

(in the formula, $R_1$ denotes an alkyl group with 1 – 5 carbon atoms, n denotes an integer of 40 – 140).

(2) A method for the preparation of a modified tissue plasminogen activator carrying, on the amino group of an amino acid residue on the tissue plasminogen activator, a polyethylene glycol unit, which is activated using an active group, by connecting the amino group of the amino acid residue on the tissue plasminogen activator to the terminal hydroxyl group of the polyethylene glycol represented by general formula (I),

$$R_1\text{-}(OCH_2CH_2)_n\text{-}OH \qquad \text{(I)}$$

(in the formula, $R_1$ denotes an alkyl group with 1 – 5 carbon atoms, n denotes an integer of 40 – 140).

3. Detailed Explanation of the Invention

Industrial field of utilization

This invention is related to a method for the preparation of a modified tissue plasminogen activator carrying, on the amino group of the amino acid residue on the tissue plasminogen activator, an activated polyethylene glycol unit, which is activated using an active group by connecting the amino group of the amino acid residue on the tissue

plasminogen activator to the terminal hydroxyl group of the polyethylene glycol. The tissue plasminogen activator of this invention maintains its basic physiological properties and has a long half-life in the blood but no antigen characteristics.

Prior art

Recently, studies on the parenteral administration of an enzyme or protein have been attracting a great deal of attention for potential treatment of various diseases.

In the treatment using an enzyme or protein, however, direct administration of an enzyme or protein into the human organism will stimulate the immune system and generate antibody, if the enzyme or protein is not originated from humans. On the other hand, if the enzyme or protein is originated from humans, it will disappear rapidly after having been introduced into the human organism.

Therefore, for potential treatment of various diseases, it is preferable to have an enzyme or protein, which is capable of maintaining its physiological properties and has a long half-life in the blood but no antigenicity. In order to achieve such a goal, extensive studies have been carried out on the chemical modification of an enzyme or protein by attaching an organic compound at the amino group of the amino acid residue on the enzyme or protein [Inada, et al., *Seikagaku (Biochemistry),* Vol. 52, No. 12, p 1225 – 1267 (1980)].

Plasminogen activators are known to have the function of dissolving blood clots and have been classified into two groups based on their immunological features, urokinase plasminogen activator and tissue plasminogen activator. These plasminogen activators are all endoproteases with a function of converting plasminogen, an inactive precursor, to active plasmin and can be used in the treatment of various diseases due to blood clots, such as cardiac infarction, pulmonary thrombosis, chest venous thrombosis, peripheral arterial thrombosis, etc.

Extensive studies have been carried out on the two commercially available protein products capable of dissolving blood clots, urokinase and streptokinase, to eliminate the antigenicity and increase the half-life in the blood, while maintaining the physiological properties.

It was reported in Japanese patent Kokai Sho 58-96026 that, after chemical modification on the lysine residue and N-terminal amino group with polyethylene glycol with an average molecular weight of 5000 using an active coupling agent, the urokinase of human origin showed an increase in the half-life in blood when administered to rabbits through intravenous injection. Moreover, in Japanese patent Kokai Sho 57-118789, it was reported that the antigenicity of streptokinase can be minimized, while the capability of dissolving blood clots can be maintained, by replacing 5 – 10% of the hydrogen atoms of the amino group in the molecule with polyethylene glycol having an average molecular weight of 750 – 10000 by a triazine ring.

Problems to be solved by the invention

Since urokinase and streptokinase have very low affinity to fibrin compared to that of the tissue plasminogen activator (abbreviated below as t-PA), it is impossible to activate selectively the plasminogen bound to the fibrin in the dissolved form. In other words, most of the plasmin generated through unlimited activation of plasminogen is neutralized before reaching any blood clots. Therefore, in order to achieve the goal of using a blood-clot-dissolving agent, a high dose must be used, which may result in side effects, such as internal bleeding, etc. In addition, streptokinase has a high antigenicity and should not be used in patients with a high antibody value.

Since t-PA has a high affinity to fibrin, the plasminogen bound to the fibrin will be selectively activated, suggesting that t-PA may provide a high efficacy for the treatment of thrombosis. However, t-PA has a complicated structure [Collen, et al., *Thromb, Haemostas,* **52**, 24 – 26 (1984)] and can be inhibited by various inhibitors present in the organism. Moreover, metabolism of t-PA itself in the organism is very fast and the half-life in the blood is only about 2 – 3 minutes [C. Korpin ger *Thromb, Haemostas,* **46,** 658 – 661 (1981)]. According to the author, when measured in New Zealand rabbits by injecting a nembutal solution into the ear veins under general anesthesia, single chain t-PA showed a short half-life of about 66 seconds and double chain t-PA showed an even shorter half-life of about 39 seconds.

To date, however, nothing has been reported on studies of the chemical modification of t-PA to improve the stability in the blood and to increase the half-life in the organism.

Therefore, it is highly desirable to increase the half-life of t-PA in the organism and eliminate the antigenicity through chemical modification of t-PA.

Techniques used for solving the problems

In order to achieve the above goal, the inventors carried out a series of studies. As a result, it was found that when polyethylene glycol with a molecular weight of 5000 was attached to the amino group of the lysine residue on the polypeptide chain of t-PA, the half-life of t-PA in blood was increased by about 8 fold, when the modified t-PA was administered to New Zealand rabbits through intravenous injection. In addition, a residual activity of 10% or higher was maintained for more than 1 hour.

This invention concerns a modified tissue plasminogen activator carrying, on the amino group of the amino acid residue on the tissue plasminogen activator, an activated polyethylene glycol unit, which is activated using an active group, by connecting the amino group of the amino acid residue on the tissue plasminogen activator to the terminal hydroxyl group of the polyethylene glycol represented by general formula (I),

$$R_1\text{-}(OCH_2CH_2)_n\text{-}OH \qquad \text{(I)}$$

(in the formula, $R_1$ denotes an alkyl group with 1 – 5 carbon atoms, n denotes an integer of 40 – 140).

In this invention, the t-PA should contain the protease part generated through culturing microorganisms or cells and have its physiological properties very close to the properties of t-PA present in tissues. In other words, the t-PA of this invention can be a product of genetic engineering or can be generated by various cell lines, including normal cell lines, such as human endothelial cells, human uterine cells, etc., as well as tumor cell lines, such as human melanoma cells, breast cancer cells, etc.

The t-PA of this invention should have the following physicochemical properties.

(1) Molecular weight: 62000 – 73000

(2) Function and substrate specificity

The enzyme is able to convert the inactive precursor, plasminogen, to plasmin, to dissolve fibrin, and shows a high affinity to fibrin compared to the commercially available enzyme, urokinase, and has a $K_m$ value of $3.6 \times 10^{-4}$ M and $V_{max}$ value of 116

pmol/min·t·U. determined with a synthetic substrate, S-2288 (H-D-Isoleucyl-L-prolyl-L-arginine-p-nitroanilide dihydrochloride, Daiichi Kagaku Co.).

(3) Optimum pH 7 – 11

(4) Stable pH 4.5 – 11

(5) Optimum temperature: 30 – 45°C

(6) Temperature stability: almost no activity loss after 90 minutes at a temperature up to 50°C

(7) UV absorption spectrum: absorption maximum at 280 nm

(8) Solubility in water or solvents: 50 μg/mL in water or aqueous salt solution, such as phosphate buffer, etc. However, a dissolution assistant is needed for other solvents.

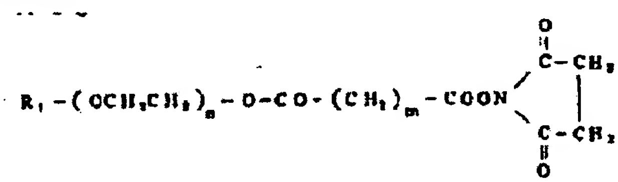
(9) State: white powder after lyophilization

(10) Color reaction: showing a pink color characteristic for glycoprotein in the PAS reaction.

(11) Isoelectric point: pH 7.5 – 8.0

In this invention, the activated polyethylene glycol (abbreviated below as activated PEG) was prepared with the known method described below.

Thus, the hydroxyl group of the polyethylene glycol (abbreviated below as PEG) represented by general formula (I) is first esterified with an aliphatic dicarboxylic acid with 3 – 6 carbon atoms. Then, the terminal carboxyl group is connected to *N*-hydroxysuccimide to form the *N*-hydroxysuccimide polyglycol represented by the following general formula:



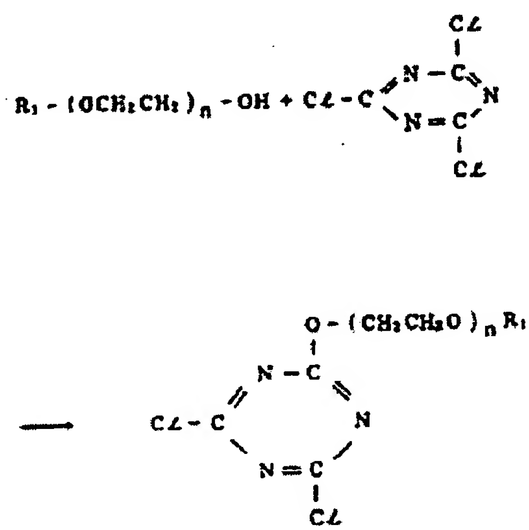
In the formula, $R_1$ denotes an alkyl group, such as a methyl group, ethyl group, propyl group, isopropyl group, butyl group, isobutyl group, or pentyl group. Particularly, the methyl group is preferable.

In the formula, the group of $-CO-(CH_2)_m-CO-$ denotes a linker residue, and m denotes an integer of 1 – 4. Good examples of the linker residue are the malonyl group

(–CO-$CH_2$-CO), succinyl group (–CO-$(CH_2)_2$-CO-), glutaryl group (–CO-$(CH_2)_3$-CO-), or adipyl group (–CO-$(CH_2)_4$-CO-), etc.

One end of the linker residue is joined to the residual oxygen atom of the terminal hydroxyl group of PEG and the other end is joined to the ε–amino group of the lysine residue or the O-amino group of the *N*-terminal on the t-PA molecule.

In a different method, a cyanuric halide, such as cyanuric chloride, etc., may also be used for the activation of PEG [Inada et al., *Immunochemistry*, 12, 899 – 902 (1975)]. The reaction is shown in the following scheme.



2-alkoxyPEG-4,6-dichloro-1,3,5-triazine
(in the scheme, $R_1$ and n have the same meanings as above)

Moreover, the terminal hydroxyl group of PEG can also be activated through the reaction with chloroacetic acid and then reacted with diazomethane to form a carboxymethyl ester, followed by the treatment with hydrazine to add an acylazide group (Japanese patent Kokoku Sho 56-23587).

In this invention, the terminal hydroxyl group of PEG can be activated with any method, as long as the activated PEG has a high reactivity and is able to react selectively under mild conditions with the ε–amino group of the lysine residue and will not cause side effects when the product enzyme is administered to humans.

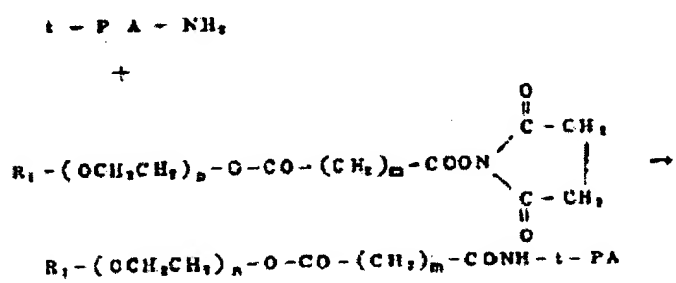
Next, the modification reaction of t-PA with the activated PEG should be carried out under the conditions which do not cause any activity loss of the t-PA.

It is known that t-PA carries a kringle region, which shows a high affinity to fibrin, as well as a serine protease part, which shows the specific activity of being able to interact with and convert plasminogen to active plasmin [Collen et al, *Nature,* **301**, 214 – 221 (1983)]. Therefore, the modification of the t-PA should not affect the functions of the two regions mentioned above.

In order to increase the half-life of t-PA in blood without affecting its activity, the modifying agent and method must be selected carefully. However, it is unavoidable to have some activity loss when using the methods currently used for the modification of an enzyme or protein to eliminate the antigenicity. For the modification of t-PA, since t-PA has a complicated structure and the local environment around the ε–amino group of the lysine residue is also variable, selective modification is extremely difficult.

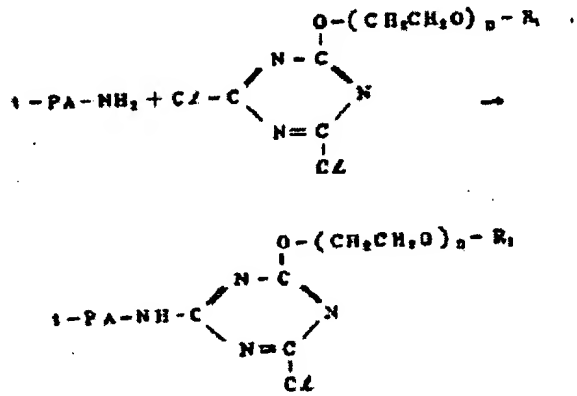
In the following, modification of t-PA using activated PEG is described in detail.

First, *N*-hydroxysuccinylimide polyglycol, $2.41 \times 10^3 - 1.27 \times 10^5$ mole, is reacted with 1 mole of t-PA in a borate buffer or phosphate buffer with pH 6.5 – 9.0, preferably 6.5 – 7.5, at room temperature. The average molecular weight of PEG should be in the range of 1900 – 5000.



The modified t-PA thus obtained can be further purified with methods commonly used for the purification of proteins without affecting the physiological activity of t-PA, including methods such as dialysis, ultrafiltration, gel filtration, etc., as well as a combination of these methods.

When using other types of activated PEG, such as 2-alkoxy PEG-4,6-dicloro-1,3,5-triazine prepared through the reaction with cyanuric chlirode, etc., the reaction of t-PA and the activated PEG is carried out in a borate buffer. When the reaction is completed, the excess amount of the activated PEG is removed and the activated t-PA is then purified with common methods.



Significance of the invention

The modified t-PA of this invention is less affected by various inhibitors present in the organism, has an increased half-life in the blood and is a very useful agent for dissolving blood clots.

The modified t-PA of this invention is more stable and capable of maintaining 15% or more of the physiological activities of t-PA, including the ability of dissolving blood clots. The half-life of the modified t-PA of this invention in the blood was increased by about 8-fold compared to the half-life of unmodified t-PA.

Moreover, while unmodified t-PA disappears from the blood in about 20 minutes, the modified t-PA of this invention is able to maintain 10% or more of its residual activities in the blood for more than 1 hour.

The modified t-PA of this invention can be used safely for the treatment of various diseases due to blood clots, such as cardiac infarction, pulmonary thrombosis, pulmonary venous thrombosis, peripheral arterial thrombosis, etc.

When the modified t-PA of this invention is used as a drug for the treatment of diseases, the modified t-PA can be administered directly into the blood, particularly at the location

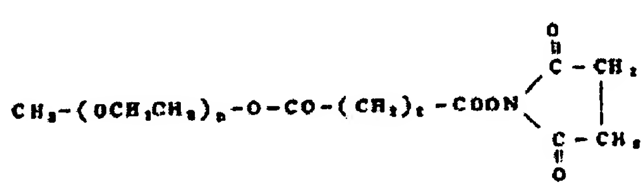
where the blood clots form. However, it is preferable to use intravenous administration of the modified t-PA in clinical practice.

The modified t-PA of this invention can be used as a composition. The composition may also contain suitable amounts of other excipients as inactive ingredients, including an isotonic agent, such as sodium chloride, mannitol, glucose, etc., a stabilizer, such as mannitol, albumin, gelatin, sodium hydrogen sulfite, etc. The dose of the modified t-PA of this invention is dependent on the body weight and age of patient, on the symptoms, severity of the disease, etc., and is usually in the range of 50 μg – 500 mg. For the intravenous administration, intravenous injection is preferable. However, intravenous infusion can also be used.

<u>Practical Examples</u>
In the following, this invention is explained in more detail with practical examples.

Practical Example 1
<u>Modification of t-PA using activated PEG derivative</u>
(1) Purification of t-PA
Human rhabdomyoma strain KYM-E (Meiji Nyunyo Co.) (abbreviated below as KYM-E) culture supernatant was purified with zinc chelate column chromatography, anti-t-PA monoclonal antibody affinity chromatography, and Cephaclear 8-200 column chromatography. The purified fraction has a protein concentration of 26.9 μg/mL dissolved in 10 mM phosphate buffer (pH 6.5) containing 0.3 M NaCl and 0.01% Tween 80.

(2) Modifying agent
*N*-Hydroxysuccinylimide polyglycol (Nippon Yushi Co.) prepared through activation of succinate monoester of PEG with an average molecular weight of 5000 using *N*-hydroxysuccinylimide (abbreviated below as activated $PEG_{5000}$) was used. The structure is as follows.



(3) Modification

The solution of the purified t-PA (molecular weight of about 70000, 3995 IU/mL), 75 mL, was dialyzed against 1 M potassium phosphate buffer (pH 7.5) at 4°C overnight to obtain a t-PA buffer solution.

Next, activated $PEG_{5000}$ (Nippon Yushi Co.), 2.4 g, was added to the buffer solution in 10 portions and the reaction was carried out at 4°C for 2 hours. The concentrations of t-PA and activated $PEG_{5000}$ in the reaction solution were $5.76 \times 10^{-5}$ and $4.37 \times 10^{-2}$ M, respectively.

Then, the reaction solution was dialyzed against 10 mM phosphate buffer (pH 7.5) containing 0.02% (w/v) Tween 80 and 0.15 M NaCl and the final volume was reduced to 9 mL. The activity of the solution obtained was 3467 IU/mL.

When the reaction was completed, the modified t-PA had a molecular weight of about 120,000 based on the high performance liquid chromatography results obtained using a Superlose 12 column (Pharmacia Co.) and protein molecular weight calibration curve. Since the molecular weight of t-PA itself was about 70,000, about 10 units of $PEG_{5000}$ were introduced onto the lysine residue and the *N* terminal amino group on the polypeptide chain of each t-PA molecule through the linker.

Practical Example 2

t-PA purified from Bowes strain (US American Diagnostica Co.) was modified under various conditions using the same activated $PEG_{5000}$ agent as in Practical Example 1. The results are shown in Table 1.

Table 1

| t-PA (double chain) | | | Activated PEG | | | Reaction conditions | | Modified t-PA average molecular weight |
|---|---|---|---|---|---|---|---|---|
| Sample | µg/mL | Molar conc. | Mean | mg | Molar conc. | °C | pH | |
| 1 | 18.3 | $2.7 \times 10^{-7}$ | 5000 | 960 | $3.2 \times 10^{-2}$ | 4 | 6.0 | 128,000 |
| 2 | 18.3 | $2.6 \times 10^{-7}$ | 5000 | 240 | $8.0 \times 10^{-3}$ | 4 | 6.5 | 115,000 |
| 3 | 18.3 | $2.7 \times 10^{-7}$ | 5000 | 240 | $8.0 \times 10^{-3}$ | 4 | 7.0 | 118,000 |
| 4 | 18.7 | $2.7 \times 10^{-7}$ | 5000 | 200 | $4.0 \times 10^{-3}$ | 4 | 7.5 | 110,000 |
| 5 | 18.3 | $2.7 \times 10^{-7}$ | 1900 | 52.5 | $7.9 \times 10^{-3}$ | 25 | 7.4 | 88,000 |

* The molecular weight of t-PA was 68,000 (Sample 1), 70000 (Sample 2), 68,000 (Sample 3), 70,000 (Sample 4), and 69,000 (Sample 5).

Test Example 1

Half-life and activity of KYM-E origin modified t-PA in blood

New Zealand white rabbits (male, body weight 2.8 – 3.2 kg) were treated with general anesthesia using Nembutal injection solution (30 mg/kg, Dainippon Seiyaku Co.) and fixed. The modified t-PA (3467 IU/mL) prepared in Practical Example 1, 2 mL, was injected into the ear vein. Then, blood samples, 2.7 mL each, were collected using sample tubes containing 0.3 mL of 3.8% sodium citrate at time points of 1, 2, 3, 4, 5, 6, 8, 10, 15, 20, 30, 45, and 60 minutes after injection. As a control sample, unmodified KYM-E origin t-PA (4550 I. U./mL) was also administered into the rabbits and blood samples, 2.7 mL each, were also collected.

The whole blood samples thus obtained, 3 mL, were centrifuged (4°C, 3500 rpm) for 10 minutes to separate the serum. The serum samples, 1 mL, were diluted 10 fold with distilled water, while cooling in an ice-bath. After the pH value was adjusted to 5.9 using 2% acetic acid, the samples were set aside at 4°C for 30 minutes and then centrifuged to separate the Euglobulin fraction as a precipitate. The supernatant was removed and the Euglobulin fraction was dissolved in buffer A (50 mM phosphate buffer containing 0.1 M NaCl, 0.25% gelatin, 0.01% Tween 80, pH 7.75).

The activity of t-PA in the Euglobulin fraction was determined with the modified fibrin clot dissolution method, originally reported by Rijkin et al. [Rijkin et al., *J. Biol. Chem.*, **256**, 7035 – 7041 (1981)]. The fibrin clot was formed by mixing 0.5 mL of 2.4 mg/mL human fibrinogen, 0.1 mL of a standard urokinase solution or Euglobulin fraction, 0.05 mL of 0.3 mg human plasminogen, and 0.05 mL of a 40 NIH units/mL thrombin solution. All sample solutions were diluted and prepared with buffer A. After adding the thrombin solution, a stopwatch was started and fibrin clot formation was carried out by incubating the mixture in a thermostat at 37°C. Then, a nylon ball with a diameter of 3.2 mm was placed on the top of the fibrin clot. The time for the nylon ball to reach the bottom of the test tube was recorded.

The fibrin clot dissolution time (second) was determined using different concentrations of urokinase. A calibration curve was prepared by plotting the urokinase unit and the dissolution time on a log-log scale.

The residual activity in the Euglobulin fraction was then calculated from the urokinase calibration curve followed by multiplying a dissolution factor and expressed in international units (IU).

The residual activity at each time point with respect to the initial activity at time zero as 100% is plotted in Figure 1.

The residual activity at time zero as 100% of modified t-PA is significantly longer than that of unmodified t-PA. The half-life of modified t-PA is about 320 seconds, which is about 8 times the 40 minute half-life of unmodified t-PA.

Modified t-PA was able to maintain 10% or higher activity for more than 60 minutes. On the other hand, however, unmodified t-PA lost its activity completely in about 20 minutes.

Therefore, it is extremely effective to maintain the activity of t-PA for a long period of time in the blood through modification using activated PEG.

Test Example 2

Half-life of t-PA modified under different conditions in blood

The t-PA samples modified under different conditions obtained in Practical Example 2 were tested for the half-life in blood with the same method as Practical Example 1. The samples were administered to white rabbits and the blood samples were collected at different time points. The Euglobulin fractions were prepared.

The t-PA activity in these Euglobulin fractions was determined with the fibrin clot dissolution method. The results obtained are shown in Table 2.

Table 2

| Sample | Residual activity % | Half-life in blood, t1/2 | Average number of PEG attached |
|---|---|---|---|
| 1 | 15.1 | 183 seconds | 12 |
| 2 | 26.4 | 192 | 9 |
| 3 | 19.3 | 174 | 10 |
| 4 | 28.1 | 260 | 8 |
| 5 | 32.9 | 136 | 10 |

As shown in Table 2, the half-life of modified t-PA in the blood is increased significantly from the 39-second half-life of unmodified t-PA in the blood. The residual activity was maintained at 15% or higher.

Test Example 3

Antigenicity of modified t-PA

Activated $PEG_{5000}$, 0.5 – 25 mg, was added as a powder to 500 μL of 0.1 M borate buffer (pH 8.5) containing 135 ng of KYM-E origin t-PA. The reaction was carried out at 4°C for 2 hours.

When the reaction was completed, the antigenicity was determined for modified t –PA and unmodified t –PA containing also activated monomethoxy PEG with the sandwich type enzyme immunoassay (abbreviated below as EIA).

In EIA, the sample solution was diluted with 10 mM phosphate buffer (pH 7.0) containing 1 mM $MgCl_2$, 0.15 M NaCl, 0.1% bovine serum album, and 0.1% $NaN_3$.

The sample, 150 μl, was incubated with 1 polystyrene bead (diameter 3.2 mm) carrying anti-t-PA antibody (rabbit IgG) at 37°C for 4 hours and then the reaction was carried out under the same conditions overnight. When the reaction was completed, the bead was washed with 0.9% aqueous NaCl solution three times and incubated with 2000 units of β–galactosidase-labeled antibody (rabbit Fab') at 37°C for 6 hours. When the reaction was completed, the bead was washed with 0.9% aqueous NaCl solution three times. The activity of the β–galactosidase loaded on the bead was determined through the reaction with 4-methylumbelliferyl-β-D-galactoside as a substrate to form 4-methylumbelliferone, followed by measuring the fluorescence intensity of the product using an excitation wavelength of 360 nm and an emission wavelength of 450 nm. The enzymatic reaction was carried out at 37°C for 20 minutes. The results are shown in Figure 2.

As shown in the figure, the antigenicity of t-PA was reduced significantly after the modification. For example, the t-PA modified with 10 mM activated PEG-E derivative lost almost 100% of its antigenicity, while the t-PA modified with 0.2 mM activated PEG-E derivative lost more than 50% of its antigenicity.

The results suggest that the modification method of this invention is very effective for minimizing the antigenicity of t-PA.

4. <u>Brief explanation of the drawings</u>

Figure 1 shows the time-dependent changes of the plasminogen activator activity in rabbit blood for the KYM-E origin unmodified and modified t-PA. Figure 2 shows the residual antigenicity determined with EIA for t-PA modified with different PEG concentrations.

Applicant: Meiji Nunyo Co., Ltd.

Figure 1

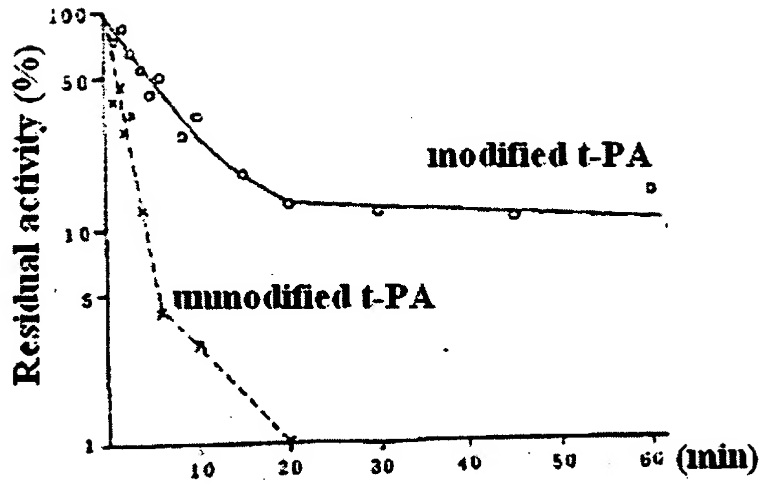



Figure 2